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Differential effect of Phenoxy Substituted Herbicide 2, 4-D on Pigments, Metabolites, and Enzyme Activities of Three Species of Cyanobacteria

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ABSTRACT

The effect of graded concentrations of common rice field herbicide (2,4-Dichlorophenoxy Acetic Acid Ethyl ester) on pigments, metabolic contents, enzyme activities and tolerance potential of cyanobacterial strains *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* was assessed and compared at periodic intervals of 4th and 16th day after treatment. Cyanobacterial strains showed progressive declination of pigments with increase in dosage of herbicides. This is evident from considerable diminution in pigments contents like chlorophyll-a (60-86%), carotenoids (64-80%), phycocyanin (65-93%), phycoerythrin (67-95%) and allophycocyanin (63-94%), metabolites like carbohydrates (65-81%), proteins (50-63%) and aminoacids (57-75%) and suppression of enzymatic activity such as nitrate reductase (62-78%), glutamine synthetase (69-97%) and Succinate dehydrogenase (73-89%). However, an increase by 15-29% in phenol content was recorded in all the test species against to herbicide doses. *W. prolifica* showed the greater tolerance towards different doses of 2,4-D than *Aulosira fertilissima* and *Anabaena fertilissima*.

Keywords: 2,4-D, pigments, metabolic contents, enzyme activity, cyanobacteria

INTRODUCTION

Effects of herbicides on non-target organisms in the soil ecosystem such as microorganisms have recently been paid great attention. Soil algae, particularly nitrogen-fixing cyanobacteria are important photosynthetic microorganisms because they contribute to soil fertility by fixing the atmospheric nitrogen [1]. The extreme sensitivity of cyanobacteria to herbicides is the main concern for a successful exploitation of these microorganisms as potential biofertiliser in rice culture agro ecosystems. Furthermore, an ideal biofertiliser strain of cyanobacteria must have the ability to tolerate or even resist to toxic actions of herbicides [2]. Many reports available indicate interaction between soil algae and herbicides, including effects of herbicides on algal growth, photosynthesis, respiration, nitrogen fixation, biochemical composition, and metabolic activities [3,4]. Nirmal Kumar and Rana [5] studied the metabolic response of *Nostoc muscuron* when treated with different doses of urea compound isoproturon. Gadkari [6] reported two to ten times higher resistance of *Nostoc muscorum* to triazine herbicides as compared with *Anabaena cylindrica*. The present study was aimed to determine the chronic effect of the herbicide 2,4-Dichlorophenoxy acetic acid commonly used in agriculture on growth or pigments, metabolic contents, enzyme activities and tolerance potential and survival of cyanobacterial strains such as *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* which occur abundantly in paddy fields and contribute to the maintenance of local soil fertility and tolerance capacity of these tested cyanobacteria against different concentrations of substituted phenoxy herbicide.

MATERIALS AND METHODS

Source of Organisms and Growth Conditions

Three species of filamentous cyanobacteria *Anabaena fertilissima* Rao, C. B., *Aulosira fertilissima* Ghose and *Westiellopsis prolifica* Janet employed in this study were obtained from the National Facility for Blue Green Algal Collections, IARI, New Delhi. The herbicide tolerance was tested by growing the cyanobacterial cultures in nitrogen-free form of BG-11 liquid medium [7] and evaluated LC_{50} by supplemented with varying concentrations of 2,4-D Ethyl ester (38 EC). All cultures were grown under controlled illumination of $40\mu Em^{-2}s^{-1}$ for 14:10 h light/ dark periods at 25±2°C temperature under aerobic and static conditions.

Herbicide Preparation

2,4-D Ethyl ester (38 EC) was prepared in stock solution and added aseptically to the culture medium to the final concentrations indicated for each treatment. Cyanobacteria showed gradual but sustainable inhibition with increasing concentrations of herbicide. 50% lysis (LC_{50}) occurred in the culture treated with 30 ppm (*Anabaena fertilissima*), 40 ppm (*Aulosira fertilissima*) and 60 ppm (*W. prolifica*). Thus, three concentrations for each species were selected for the

present investigation to carry out the response to *Anabaena fertilissima, Aulosira fertilissima,* and *W. prolifica*. One concentration is LC_{50} concentration, another concentration is lower and the third concentration is higher to LC_{50} concentration. Therefore, different concentrations like 15, 30 and 60 ppm for *Anabaena fertilissima*, 20, 40, and 80 ppm for *Aulosira fertilissima* 30, 60, and 120 ppm for *W. prolifica* were selected to carry out present study.

Analytical Methods

Chlorophyll content was determined spectrophotometrically according to Jeffrey and Humphrey [8]. The content of carotenoids was determined according to Parsons and Strickland [9], while phycobilinpigments like phycocyanin, phycoerythrin and allophycocyanin were estimated according to Bennett and Bogorad [10]. Total carbohydrate was estimated by Hedge and Hofreiter [11], total protein content was determined by Lowry *et al.* method [12], total amino acid by Lee and Takahasi [13] and phenol estimation was carried out by Malick and Singh [14]. For the estimation of cellular nitrate reductase activity (NR) an in -situ assay procedure of Sempruch *et al.* [15] was used. Estimation of glutamine synthetase (GS) activity was done by γ-glutamyl transferase as described by Pamiljans *et al.* [16]. The estimation of in vivo succinate dehydrogenase (SDH) activity was measured by the method of Kun and Abood [17]. Each experiment was conducted in replicates of three and their ±SD values were calculated.

RESULTS AND DISCUSSION

Herbicides are reported to affect cyanobacteria in various ways [18] and have differential effects on various metabolic processes and the sensitivity of the strain varies depending upon the species, kind of herbicides and chemical formulations [19]. It had been reported that sensitivity to herbicides varied with species [20].

Inhibitory Effect of 2,4-D on Pigments

2,4-D Ethyl ester showed the most deleterious effect on the growth of *Anabaena fertilissima* among the three tested cyanobacterial species. Immediately after 4-days of exposure to high herbicide levels, Chlorophyll-a contents showed a significant decrease of 36% at 60 ppm in *Anabaena fertilissima*, 30% at 80 ppm in *Aulosira fertilissima* and 23% at 120 ppm in *W. prolifica* relative to control. The maximum reduction in chlorophyll a content was recorded in *Anabaena fertilissima* (86% at 60 ppm), and the lowest in *W. prolifica* (60% at 120 ppm) followed by *Aulosira fertilissima* (77% at 80 ppm) by the end of 16^{th} day of growth (Fig.1). In present study, chlorophyll synthesis was found to be severely affected by the graded concentrations of 2,4-D and lysis of cultures occurred between 60-120 mg L^{-1} of 2,4-D in different isolates. Similar observations were also made by Goyal *et al.* [21] that stimulation in the chlorophyll a synthesis upto 10 mg L^{-1} and survival upto 100 mg L^{-1} of Arozin in *A. variabilis* ARM 310.

Carotenoid content in all three selected strains was affected, in a time-dose response manner, in cultures treated with 2, 4-D. After 4 days of incubation, the carotenoids values in *Anabaena fertilissima* were reduced by 32 % at 60 ppm, whereas in *Aulosira fertilissima* and *Westilliopsis prolifica*, carotenoids were reduced by 22 % at 80 ppm and 15 % at 120 ppm, respectively (Fig. 2). At the end of the experiment after 16 days, carotenoids in *Anabaena fertilissima* were depressed by 80% at 60 ppm. However, as compared to *Anabaena fertilissima* less reduction of carotenoids was observed in *W. prolifica* by 64% (at 120 ppm) followed by *Aulosira fertilissima* where the values were reduced by 72% relative to control. After surveying the effects of several carotenoid-inhibiting herbicides, Sandmann *et al.* [22] noted that inhibition of carotenoid formation in *Scenedesmus acutus*, was twice as great as inhibition of chlorophyll formation. In 2,4-D -treated cultures the phycobilin pigments were more adversely affected than chlorophyll a in all three cyanobateria. Exposure of *Anabaena fertilissima, Aulosira fertilissima* and *W. prolifica* cells to respective higher concentrations of chlorophenoxy herbicide showed concentration induced inhibition of phycocyanin, phycoerythrin and allophycocyanin by 53%, 60%, and 35% at 60 ppm in *Anabaena fertilissima*, 31%, 26% and 28% at 80 ppm in *Aulosira fertilissima* and 21%, 23% and 26% *W. prolifica*, respectively after 4 days of exposure (Fig. 3 to 5). Moreover, on day 16 of growth, phycocyanin was significantly reduced by 93% at highest graded concentration (60 ppm) in *Anabaena fertilissima*, whereas in *Aulosira fertilissima* and *W. prolifica* it was dejected by 81% at 80 ppm and 65% at 120 ppm, respectively. Likewise, phycoerythrin and allophycocyanin (mg/20 ml) were markedly reduced by 95% and 94%, respectively in *Anabaena fertilissima*, while 79% and 76% in *Aulosira fertilissima.* As compared to other two strains low level of reduction was noticed in *W. prolifica*, where phycoerythrin was reduced by 67% and allophycocyanin by 63% at 120 ppm. The highest reduction of phycobilin pigments suggested that under herbicide stress there was a diversion to meet the nitrogen demand possibly through the induction of proteolytic enzymes [23].

Metabolites Exposed To Chlorophenoxy Herbicide (2,4-D)

The release of carbohydrates was found to be higher in untreated cells in all tested periodic intervals in three

species. *W. prolifica* was found to be more defiant as compared to other two strains. On day 4th of incubation release of carbohydrates in *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica* was suppressed by 48% at 60 ppm, 34% at 80 ppm and 32% at 120 ppm, respectively (Fig. 6). In *Anabaena fertilissima* 81% decrease in carbohydrate content was observed at 60 ppm, whereas in *Aulosira fertilissima* and *W. prolifica* it was 70% at 80 ppm and 65% at 120 ppm after 16 days of incubation (Fig.6). Galhano *et al.* [24] verified decrease in total carbohydrates from 0 to 24 h in all bentazon treated cultures in a time-dose response manner.

Amino acids and proteins were also used to observe the effect of herbicide toxicity to *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica*. 2,4-D, an inhibitor of protein synthesis showed a more marked suppression of total protein content than of amino acids. However, slight increase at lower concentrations of three species was recorded after 4 days of inoculation, which was succeeded by a period of depression. Similar results were reported by Nirmal Kumar [25] in *Anabaena* sp.310 treated with isoproturon. Significant reduction in protein content was recorded at higher concentrations in all the three strains and a reduction of 63% at 60 ppm, 56% at 80 ppm and 50% at 120 ppm was recorded in *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica*, respectively by the end of day 16 of growth (Fig. 7). Babu *et al.* [26] observed low protein content of lindane treated cell, which was believed to be a result of higher protease activity, retarded growth, and decreased carbon and nitrogen assimilation under stress conditions.
Amino acids followed a similar trend of inhibition without any stimulation even after 4- days. Amino acid content was decreased by 40% at 60 ppm in *Anabaena fertilissima*, 27% at 80 ppm in *Aulosira fertilissima* and 16% at 120 ppm in *W. prolifica* after 4 days of herbicide exposure. After incubation of 16 days *Anabaena fertilissima* showed maximum inhibition (75% at 60 ppm) of amino acid content followed by *Aulosira fertilissima* (66% at 80 ppm), and *W. prolifica* (57% at 120 ppm) (Fig. 8). Lakshmi and Annamalai [27] have reported the inhibition of amino acids under Divap 100 stress.

The release of phenols was slightly but significantly stimulated after 4-days of exposure and was higher in all treated cultures as compare to untreated cultures (control) in all the test species. On day 16 of growth, phenol (mg/20ml) was significantly increased by 29% at 60 ppm, 18% at 80 ppm and 15% at 120 ppm from an initial level in *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica*, respectively (Fig. 9). Nirmal Kumar and Rita Kumar [28] also substantiated the findings that phenols could act as protectants by the organisms under stress or drought conditions and probably accumulation of phenolic compound of treated herbicide.

Enzymatic Activities Under Herbicide (2,4-D) Stress

2,4-D Ethyl ester caused a progressive decrease in invitro NR activity with its lethal concentration. After 4 days, NR activity in *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica* incubated with 60, 80 and 120 ppm of 2,4-D, decreased by 43%, 38% and 32%, respectively whereas 2,4-D treatment for 16 days resulted in 78% loss of nitrate reductase activity in *Anabaena fertilissima* at 60 ppm, 73% at 80 ppm in *Aulosira fertilissima* and 62% at 120 ppm in *W. prolifica* (Fig.10). Thus, it is quite revealed that this amount of herbicide would affect the NR activity of beneficial species of cyanobacteria which are so important to the nitrogen economy of wetland rice production and similar results was also reported by Singh and Tiwar [29].

In case of *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica*, 2,4-D suppressed the in vitro GS activity in a time-dose response manner (Fig.11). After 4-days, *Anabaena fertilissima* showed highest sensitivity towards tested herbicide where the in vitro GS activity was reduced to about 51% of the control at 60 ppm as compared to *Aulosira fertilissima* (43% at 80 ppm) and *W. prolifica* (40% at 120 ppm), whereas after 16days of growth, GS was

suppressed by 97% at 60 ppm, 76% at 80 ppm and 69% at 120 ppm. The suppression of GS activity in herbicide-treated culture of *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica* may be related to the suppressed NR activity under similar conditions. In *Gloeocaspa* sp., GS was reported to be more active under the nitrogen-fixing conditions [30] and the activation of GS gene (gln A) requires the nitrogen fixing conditions [31].

The suppression of succinate dehydrogenase activity by 2,4-D was more prominent as the herbicide treatment for 4 days resulted in loss of 62% at 60 ppm in *Anabaena fertilissima* followed by *Aulosira fertilissima* (54% at 80 ppm) and *Westiellopsis prolifica* (40% at 120 ppm). Parallel experiments after 16 days on *Anabaena fertilissima* showed the suppression of SDH by 89% at 60 ppm, whereas in *Aulosira fertilissima* and *Westiellopsis prolifica* it was reduced by 83% at 80 ppm and 73% at 120 ppm, respectively (Fig.12). Similarly, inhibition of succinate dehydrogenase due to fungitoxicity toward *Rhizoctonia solani* (Kuhn) was reported by Phillips and Rejda-Heath [32].

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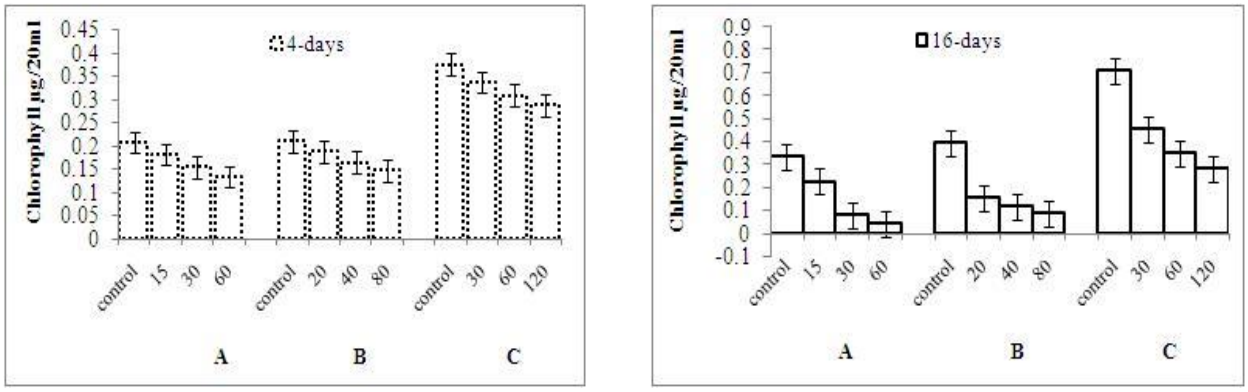

Fig. 1 Effect of 2,4-D on chlorophyll-a content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

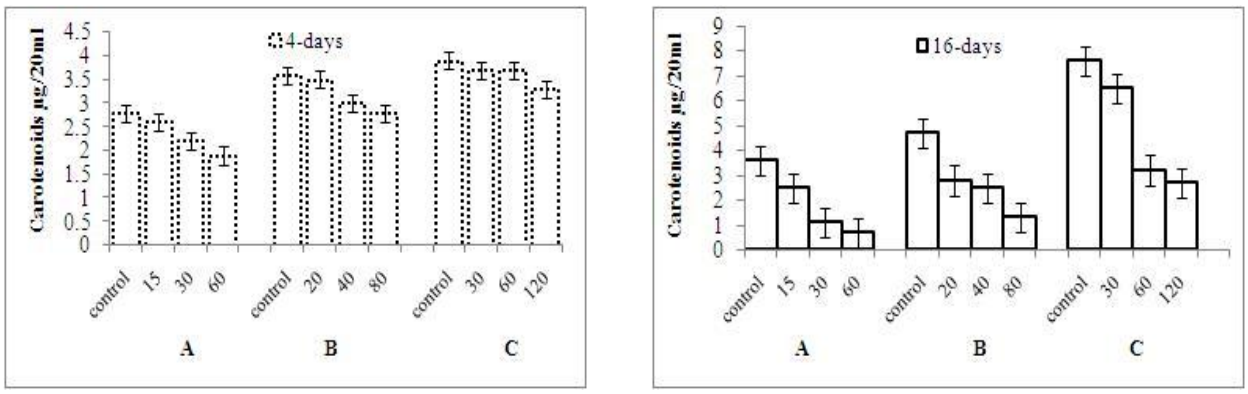

Fig. 2 Effect of 2,4-D on carotenoid content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

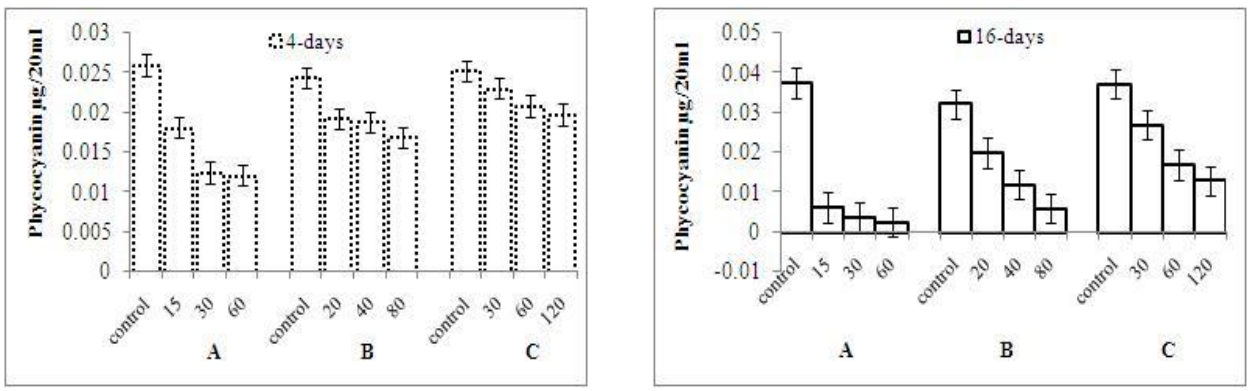

Fig. 3 Effect of 2,4-D on phycocyanin content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

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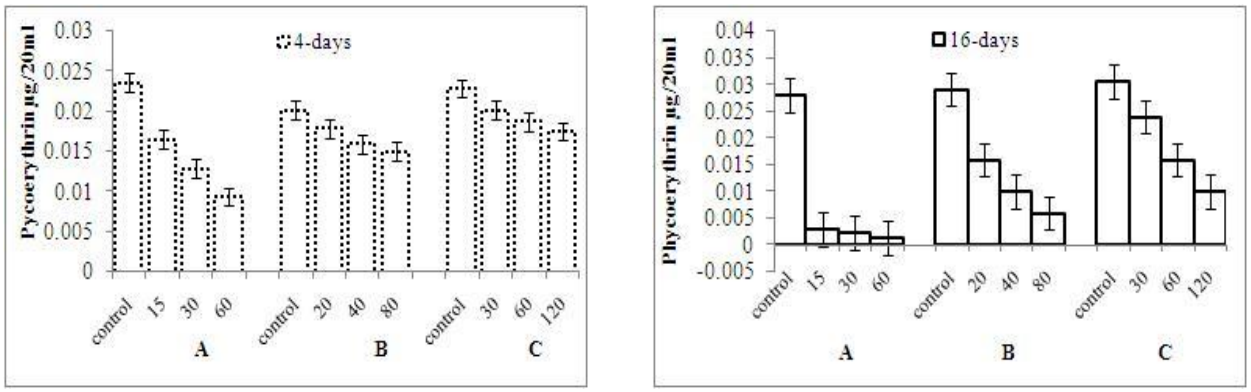

Fig. 4 Effect of 2,4-D on phycoerythrin content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

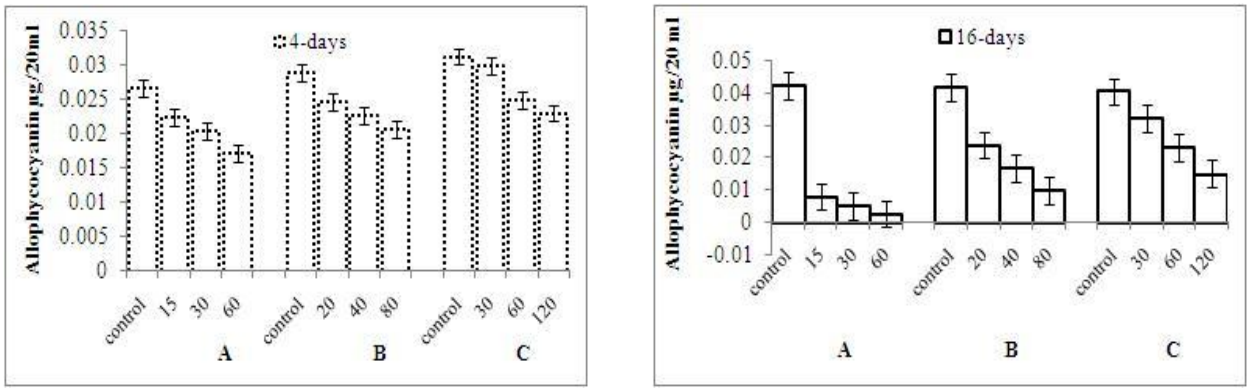

Fig. 5 Effect of 2,4-D on allophycocyanin content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

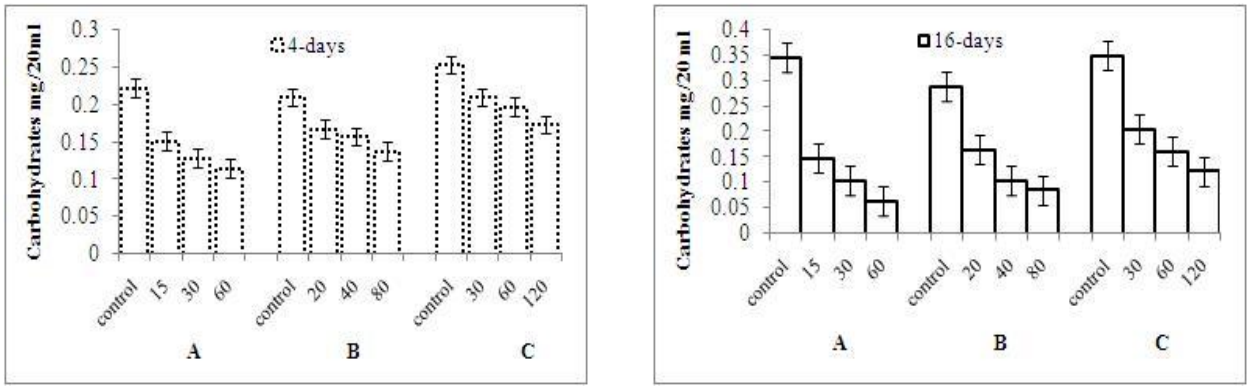

Fig. 6 Effect of 2,4-D on carbohydrate content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

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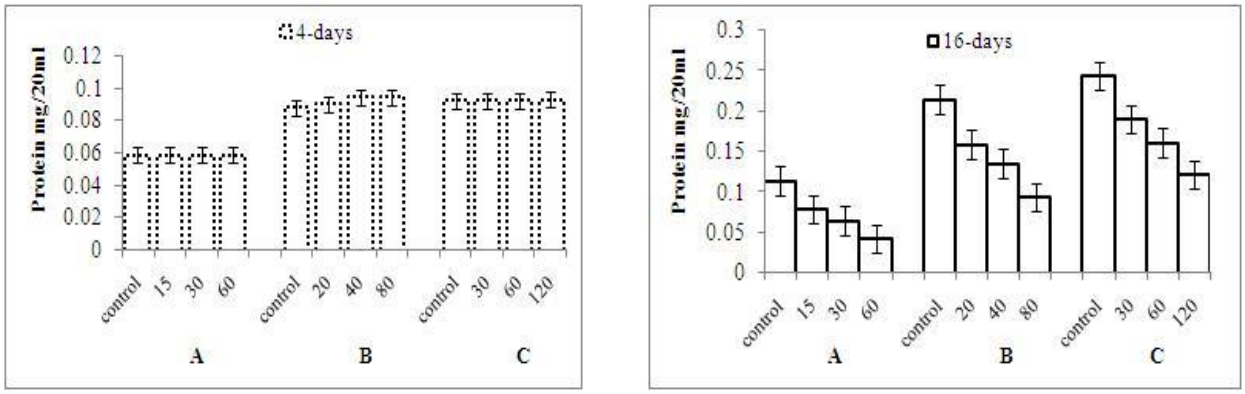

Fig. 7 Effect of 2,4-D on protein content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

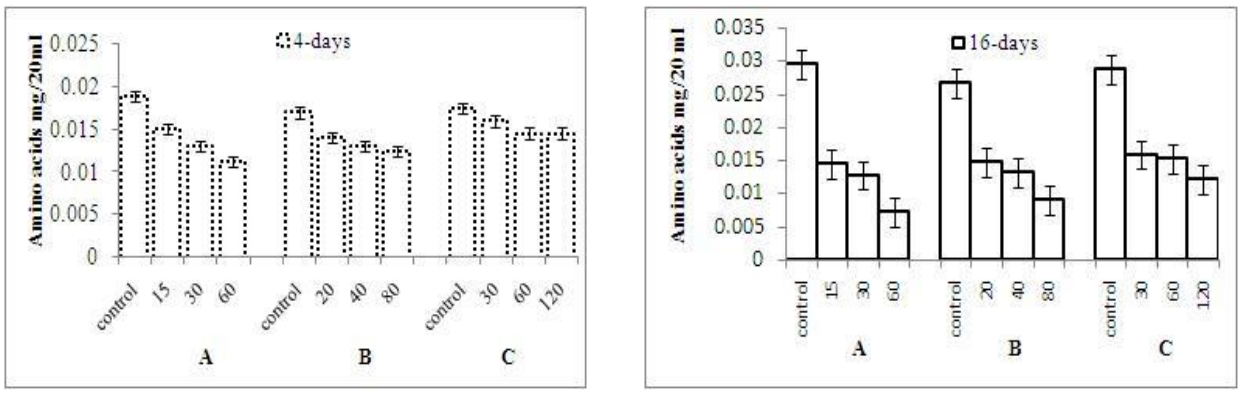

Fig. 8 Effect of 2,4-D on amino acid content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

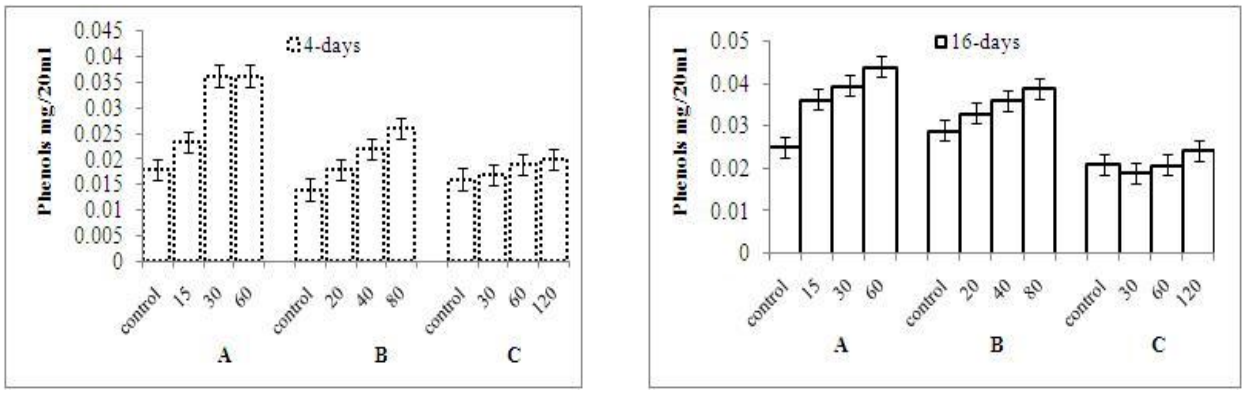

Fig. 9 Effect of 2,4-D on phenols content of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

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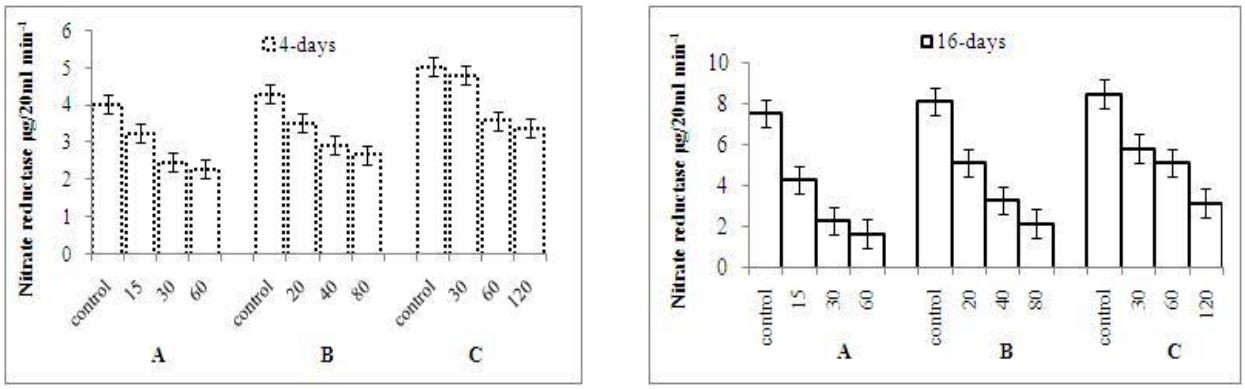

Fig. 10 Effect of 2,4-D on NR activity of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

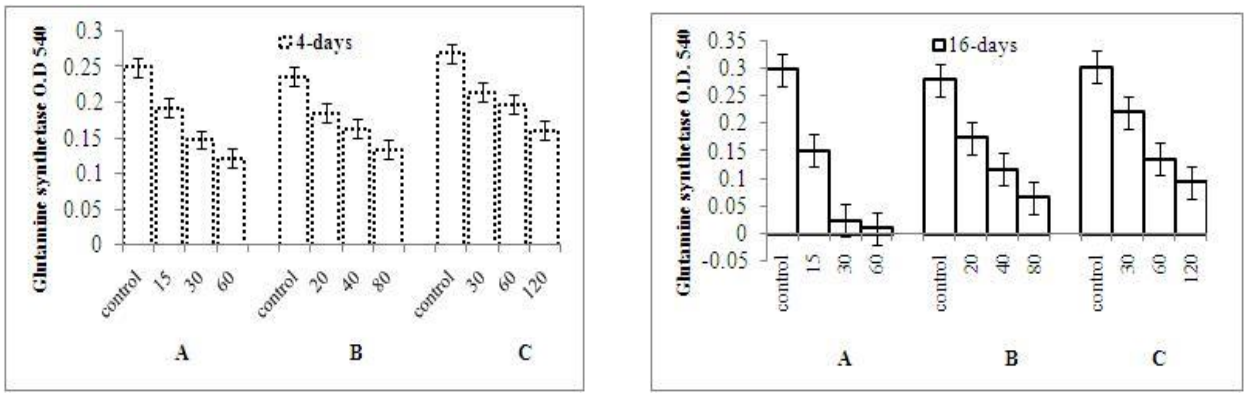

Fig. 11 Effect of 2,4-D on GS activity of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

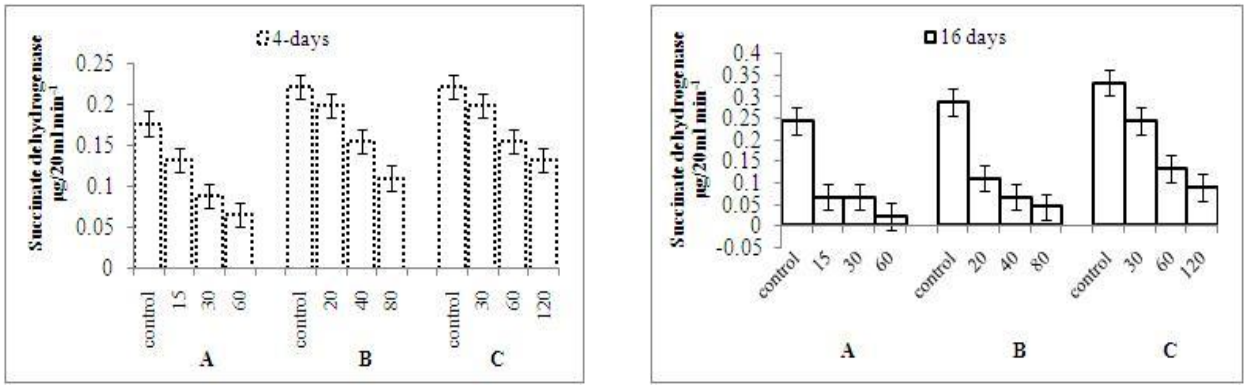

Fig.12 Effect of 2,4-D on GS activity of *Anabaena fertilissima* (A), *Aulosira fertilissima* (B) and *Westiellopsis prolifica* (C) at their respective concentrations on 4th day (left) and 16th day (right)

CONCLUSION

The residual effects of herbicides will have an adverse effect on the nitrogen-fixing potential of cyanobacteria in paddy fields. In the present study 2,4-D Ethyl ester was found to be toxic to the cyanobacteria even at lower concentrations. However, among the test organisms, *W. prolifica* showed maximum tolerance towards 2,4-D Ethyl ester than *Aulosira fertilissima* and *Anabaena fertilissima*. The higher degree of tolerance may be attributed to the capacity of the organism to accumulate this chemical in their cells at concentration several fold higher than the surrounding concentration.

For a better understanding of the mechanism of tolerance, biotransformation or intoxicants of this phenoxy compounds on these selected species can be undertaken. Nevertheless, we should take into account the species differences and the strain differences in the susceptibility when assessing the toxicity of chemicals.

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